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EFFECTS OF HEAT SHOCK AND HYPOXIA ON PROTEIN SYNTHESIS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) CELLS

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Summary

We examined the effects of heat stress (from 18 °C to 26 °C) and low oxygen tension (1 % O_2=1 kPa) on protein synthesis in primary cultures of hepatocytes, gill epithelial cells and fibroblast-like RTG-2 cells of rainbow trout *Oncorhynchus mykiss*. All these cell types displayed elevated levels of 67, 69 and 92 kDa proteins, whereas a 104 kDa protein was induced only in RTG-2 cells. Hypoxia induced a cell-type-specific response, increasing the synthesis of 36, 39 and 51 kDa proteins in the gill epithelial cells. The regulation of the heat-shock response in fish hepatocytes showed that an HSF1-like factor is involved in the transcriptional induction of the *hsp70* gene. Consequently, there was a pronounced accumulation of *hsp70* mRNA. Furthermore, the kinetics of activation of DNA binding and the increase in *hsp70* gene expression showed a remarkable correlation, indicating that *hsp70* expression is regulated at the transcriptional level in these trout cells.

Key words: heat-shock transcription factor, Hsp70, hypoxia, pH, protein synthesis, rainbow trout, *Oncorhynchus mykiss*, cellular stress response, gene expression.

Introduction

Fish, especially in the freshwater environment, are subject to marked fluctuations in both ambient temperature and oxygen availability. As in other organisms, elevated temperatures induce the synthesis of specific heat-shock proteins, Hsps (Kothary and Candido, 1982; Morimoto *et al.* 1990; Currie and Tufts, 1997; Iwama *et al.* 1998) in fish. The Hsps are divided into several protein families: Hsp104, Hsp90, Hsp70, Hsp60/GroEL and small heat-shock proteins defined by their molecular mass. All the major Hsp families suppress irreversible unfolding reactions of other proteins and thus enhance survival upon heat stress (Buchner, 1996). The induction of Hsps also occurs in fish exposed to bleached kraft pulp mill effluents (Vijayan *et al.* 1998) and to several environmental chemicals, such as arsenite (Kothary and Candido, 1982; Kothary *et al.* 1984; Grosvik and Goksoyr, 1996), to metals, such as zinc, cadmium and copper (Heikkila *et al.* 1982; Misra *et al.* 1989; Ryan and Hightower, 1994; Sanders *et al.* 1995; Grosvik and Goksoyr, 1996), and to certain polycyclic hydrocarbons (Grosvik and Goksoyr, 1996; Vijayan *et al.* 1997).

Although several environmental contaminants induce the heat-shock response in fish and although fish are faced with large fluctuations in temperature, the regulation of the heat-shock response in fish has been little studied. In many organisms, the transcription of heat-shock genes is regulated by heat-shock transcription factors (HSFs). Several factors exist and they can carry out different functions. For example, in mammalian cells, HSF1 activates expression of the heat-shock gene in response to stress (Baler *et al.* 1993; Sarge *et al.* 1993), HSF2 regulates the synthesis of heat-shock proteins during differentiation and development (Sistonen *et al.* 1992; Mezger *et al.* 1994) and HSF4 appears to function as a repressor of genes encoding Hsps (Nakai *et al.* 1997). In response to stress, HSF1 trimerizes, acquires DNA-binding activity, translocates into the nucleus and becomes hyperphosphorylated, which has been suggested to regulate the transcription of *hsp* genes (Morimoto, 1993; Sarge *et al.* 1993; Cotto *et al.* 1996). Like Hsps, the distinct isoforms of HSFs are expressed in a cell-type-specific and tissue-specific manner (Fiorenza *et al.* 1995; Goodson *et al.* 1995). As yet, no HSF has been characterized in fish.

One of the conditions inducing the synthesis of heat-shock proteins in mammals is hypoxia/anoxia, although it now appears that physiologically relevant levels of hypoxia induce a regulatory pathway different from that of heat shock and that a different set of genes is induced (Zimmerman *et al.* 1991; Graven *et al.* 1993; Tucci *et al.* 1996; Graven and Farber, 1997). Although hypoxia commonly occurs in the aquatic environment, the regulation of gene expression in fish under hypoxic conditions has been little studied. Notably, however,

the isoenzyme pattern of lactate dehydrogenase is markedly altered under hypoxic conditions (Almeida-Val *et al.* 1995).

We have initiated investigations into the regulation of gene expression in response to environmental changes, especially temperature and oxygen availability. In this study, we have compared the patterns of induction of proteins in response to elevated temperature and lowered oxygen tension using rainbow trout (*Oncorhynchus mykiss*) cells of various types, i.e. primary cultures of hepatocytes and gill epithelial cells, as well as RTG-2 cells, a cell line originating from rainbow trout gonadal fibroblasts. The use of different cell types enabled us to evaluate the tissue specificity of the responses. Studying the effect of hypoxia and temperature elevation made it possible to determine whether different sets of proteins are induced by these environmental perturbations.

Materials and methods

Primary cell cultures

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from a commercial fish farm and were kept in aquaria at 13.5±1 °C under a 11 h:13 h dark:light photoperiod with constant aeration and water circulation for at least 1 week before cell isolation. Hepatocytes were isolated using a two-step collagenase perfusion method (Seglen, 1976) as modified by Råbergh *et al.* (1992, 1995). Briefly, after killing and opening the fish, the portal vein was cannulated and the liver perfused first with a Ca^{2+}-free perfusion buffer (PBS, plus 0.5 mmol l^{-1} EGTA and 25 mmol l^{-1} Tricine, pH 7.5) to flush away the blood and then with collagenase buffer (PBS plus 50 mmol l^{-1} Hepes, 0.5 mmol l^{-1} $CaCl_2{\cdot}2H_2O$ and 0.05 % collagenase type IV, Sigma). The liver was removed and placed in a Petri dish, and the cells were dispersed. The cells were filtered and washed once with Eagle's minimum essential culture medium (Eagle's MEM, Gibco BRL) supplemented with 50 units ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (Gibco BRL), 2 mmol l^{-1} L-glutamine (Gibco BRL) and 25 mmol l^{-1} Hepes, pH 7.5. Viability was measured by Trypan Blue exclusion (0.4 % w/v) and exceeded 95 % in all cell preparations. Cells were cultured on poly-L-lysine-coated (Sigma) culture dishes (Råbergh *et al.*1995) at 18 °C under atmospheric air. Cells were used for the experiments within 48 h.

Gill epithelial cell isolations were performed according to a method modified from those of Pärt *et al.* (1993) and Lilius *et al.* (1995). Fish were killed by a blow to the head and opened ventrally, and 100 μl of heparin (25 000 i.u. ml^{-1}) (Leiras, Turku, Finland) was injected into the heart ventricle. The bulbus arteriosus was cannulated, and the dorsal aorta and sinus venosus were cut open to allow a free flow of perfusion buffer (PBS plus 50 i.u. heparin ml^{-1}) through the gills. After the perfusion, gill filaments were removed and washed three times with PBS. The cells were trypsinized and filtered as described previously (Pärt *et al.* 1993; Lilius *et al.* 1995). Epithelial cells were separated from other cell types using a Percoll (Pharmacia, Uppsala, Sweden) gradient (Lilius *et al.* 1995). Cell preparations with a viability exceeding 90 % were cultured in Eagle's MEM supplemented with 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 200 μg ml^{-1} gentamicin (Sigma Chemicals Inc., USA), 2 mmol l^{-1} L-glutamine, 25 mmol l^{-1} Hepes and 5 % foetal bovine serum (FBS; National Veterinary Institute, Uppsala, Sweden), pH 7.5 (18 °C). The medium was changed at 24 h and every 48 h thereafter. Exposures to stress were carried out after culturing for 5–8 days.

Established RTG-2 cells (American Type Culture Collection: ATCC CCL55; Wolf and Quimbly, 1962), which are immortalized rainbow trout gonadal fibroblasts, were grown to confluency in Eagle's MEM containing 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mmol l^{-1} L-glutamine, 25 mmol l^{-1} Hepes and 10 % FBS, pH 7.5 (18 °C).

Exposure to heat shock and hypoxia

For the heat-shock treatments, culture dishes containing hepatocytes, gill epithelial cells or RTG-2 cells were sealed with Parafilm and immersed in a water bath at 26 °C for 2 h. For hypoxia, cells were placed for 24 h into a sealed chamber (18 °C) which was gassed with a humidified gas mixture containing 1 % O_2 and 5 % CO_2, balanced with nitrogen. The CO_2 tension is higher than normally encountered by fish cells, but was used in these experiments to maintain the extracellular pH at a stable value of 7.5. Samples were taken 0, 1 and 2 h after heat-shock and hypoxia exposure; for northern blot analysis, samples were also taken 4 and 8 h (results not shown) after heat shock. After treatment, cells were immediately frozen in liquid nitrogen and stored at −70 °C until used. Whole-cell extracts were prepared from the samples as previously described (Mosser *et al.* 1988). Briefly, cell pellets which were kept on ice throughout the procedure were resuspended in buffer containing 25 % glycerol (v/v), 420 mmol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} EDTA, 20 mmol l^{-1} Hepes, 0.5 mmol l^{-1} phenylmethylsulphonyl fluoride and 0.5 mmol l^{-1} dithiothreitol and centrifuged for 15 min (4 °C, 14 000 ***g***). The supernatants were collected and stored at −70 °C. The protein concentration of the whole-cell extracts was determined using the Bio-Rad protein assay according to the manufacturer's instructions.

Metabolic labelling with [^{35}S]methionine

Metabolic labelling of cells during the heat-shock or hypoxia treatment was carried out as described above, but the medium was changed to a methionine-free medium (Sigma) to which 1.1 MBq of [^{35}S]methionine (43.5 TBq $mmol^{-1}$, Dupont-NEN) had been added before exposure. The radioactivity of every sample was determined, and equal amounts of radioactivity were loaded onto a 10 % SDS–PAGE gel. After separation at room temperature (20–22 °C) for 5 h at 150 V, the gel was fixed with 10 % acetic acid and 50 % methanol. Thereafter, the signal was amplified with a fluorographic reagent (Amplify; Amersham Life Science, Buckinghamshire, UK), according to the manufacturer's instructions, and visualized by exposure to X-ray film (Kodak X-OMAT MR) at room temperature. Quantification of the protein bands was performed using a

computerized image analysis system (Microcomputer Imaging Device version M4, Imaging Research Inc.). Actin was used for normalization of the data.

Gel mobility shift assay

Gel mobility shift assays were carried out on 25 μg of whole-cell protein. Samples were incubated for 20 min (at room temperature) with a reaction mixture containing γ-^{32}P-labelled HSE oligonucleotide, 5′-GCCTCGAATGTTCGCGAAGTTT-3′, modified from Zandi *et al.* (1997), corresponding to a sequence in the *Drosophila hsp70* promoter. The synthetic oligonucleotide was γ-^{32}P-labelled with T4 polynucleotide kinase (Promega). The samples were loaded onto a native 4 % PAGE gel (at room temperature) and run for 1.5 h at 150 V, as described previously (Mosser *et al.* 1988). The gel was dried under vacuum, and proteins were visualized by exposure to X-ray film (Kodak X-OMAT AR), at −70 °C. In competition experiments, an oligonucleotide from the promoter region of the thioredoxin gene (*TRX*) was used as a non-specific oligonucleotide (Taniguchi *et al.* 1996). A perturbation assay was performed using polyclonal antibodies against murine HSF1 and HSF2 (Sarge *et al.* 1993). The antibodies, at varying dilutions, were incubated with whole-cell extracts for 15 min (at room temperature) prior to the binding reaction to determine whether the electrophoretic mobility of the samples was affected by complex formation with the HSF antibody.

Northern blot analysis

Total RNA was isolated from hepatocytes by a one-step acid guanidium thiocyanate and phenol–chloroform method (Chomczynski and Sacchi, 1987) using RNAzol B (TEL-TEST, Inc., Friendswood, Texas, USA). For northern blot analysis, 10 μg of total RNA was separated on a 1.2 % agarose–formaldehyde gel for 2.5 h at 85 V, transferred to nylon membrane (Hybond-N, Amersham Corp.) and hybridized at 42 °C with a [α-^{32}P]dCTP-labelled plasmid specific for human *hsp70* (Wu *et al.* 1985). The plasmid DNA was radiolabelled using a nick translation system (Promega). After hybridization, filters were washed for 20 min with 2×SSC plus 0.1 % sodium dodecylsulphate (SDS, at room temperature), for 20 min with 1×SSC plus 0.1 % SDS (at 42 °C) and for 10 min with 0.1×SSC plus 0.1 % SDS (at room temperature) and visualized by autoradiography (Kodak X-OMAT AR). Data were normalized to the signal for ribosomal subunit 28 (28S).

Statistics

The Wilcoxon matched-pairs signed-ranks test of Statistica Software (StatSoft Inc., Tulsa, Oklahoma, USA) was used to determine significant (P<0.05) differences in protein synthesis between controls and treatments.

Results

Protein synthesis after heat shock and hypoxia

Using metabolic labelling, we were able to investigate how the pattern of protein synthesis changed in response to elevated temperature or to hypoxic conditions. In all the cell types studied (hepatocytes, gill epithelial cells and RTG-2 cells), heat-shock treatment induced the synthesis of several proteins, as demonstrated by the incorporation of radioactive methionine into protein (Fig. 1A). There was extensive synthesis of 67 and 69 kDa proteins representing members of the Hsp70 family. It appeared that the major inducible Hsp70 protein may be different in hepatocytes from that observed in gill epithelial cells or RTG-2 cells, suggesting a tissue-specific response as indicated by the quantitative differences in the induction of the 67 and 69 kDa proteins (Fig. 1B). A pronounced increase in synthesis of one member of the Hsp90 family, with a molecular mass of approximately 92 kDa, was observed in all cell types studied (Fig. 1A). In contrast, a small increase in the synthesis of a 104 kDa protein was detected in RTG-2 cells, but not in hepatocytes or gill epithelial cells. In this study, we did not focus on the downregulation of protein synthesis. However, it appears that the synthesis of some proteins was downregulated (Fig. 1A).

Fig. 2A shows the electrophoretic pattern of gill epithelial cells in control cells and in cells exposed to 24 h of hypoxia immediately after the hypoxic period (0 h) and after 2 h of recovery in normoxia. At both times, hypoxia induced the production of proteins with molecular masses of 36, 39 and 51 kDa. Although induction of all these proteins could be detected both in samples taken immediately after the treatment and in samples taken after short recovery periods (1 and 2 h of recovery), it appeared that the synthesis of a 36 kDa protein increased with time during the recovery period (Fig. 2B). In some cell preparations, there was also increased synthesis of proteins that probably represent the Hsp70 family (Fig. 2A). Hypoxia did not affect the protein synthesis patterns of hepatocytes or RTG-2 cells (results not shown). To investigate whether the hypoxia-induced protein synthesis could be due to a change in intracellular pH associated with hypoxia, we determined whether varying the pH over a physiologically relevant range (pH 6.5–8.0) affected protein synthesis. Altering the pH, however, had no effect on the protein synthesis patterns (data not shown), indicating that reduced O_2 tension is the most likely reason for inducing the synthesis of the 36, 39 and 51 kDa proteins.

Regulation of the heat-shock response

Role of a heat-shock factor

Since several proteins belonging to the major Hsp families were induced in all the fish cell types in response to elevated temperature, we chose to investigate the regulation of the response in more detail in one cell model system, the hepatocytes. To examine whether the response was regulated by transcription factors similar to those found in other organisms, we incubated hepatocyte extracts together with an oligonucleotide containing *Drosophila* HSE, the specific sequence in the *hsp70* promoter region that binds regulatory factors. The results show that one or several proteins in the cell extracts bind strongly to the HSE oligonucleotide, as seen by a reduction in the electrophoretic mobility of the labelled

oligonucleotide (Fig. 3A). Binding was detected immediately after the heat shock, and it gradually disappeared within 4 h. To examine the specificity of protein–HSE complex formation, a competition experiment with an excess of unlabelled oligonucleotide was carried out. The results showed that binding occurred specifically to the HSE, since the complex was not detected in the presence of unlabelled HSE oligonucleotide and since the oligonucleotide representing thioredoxin did not affect formation of the complex (Fig. 3B). We next determined whether the binding activity was due to a

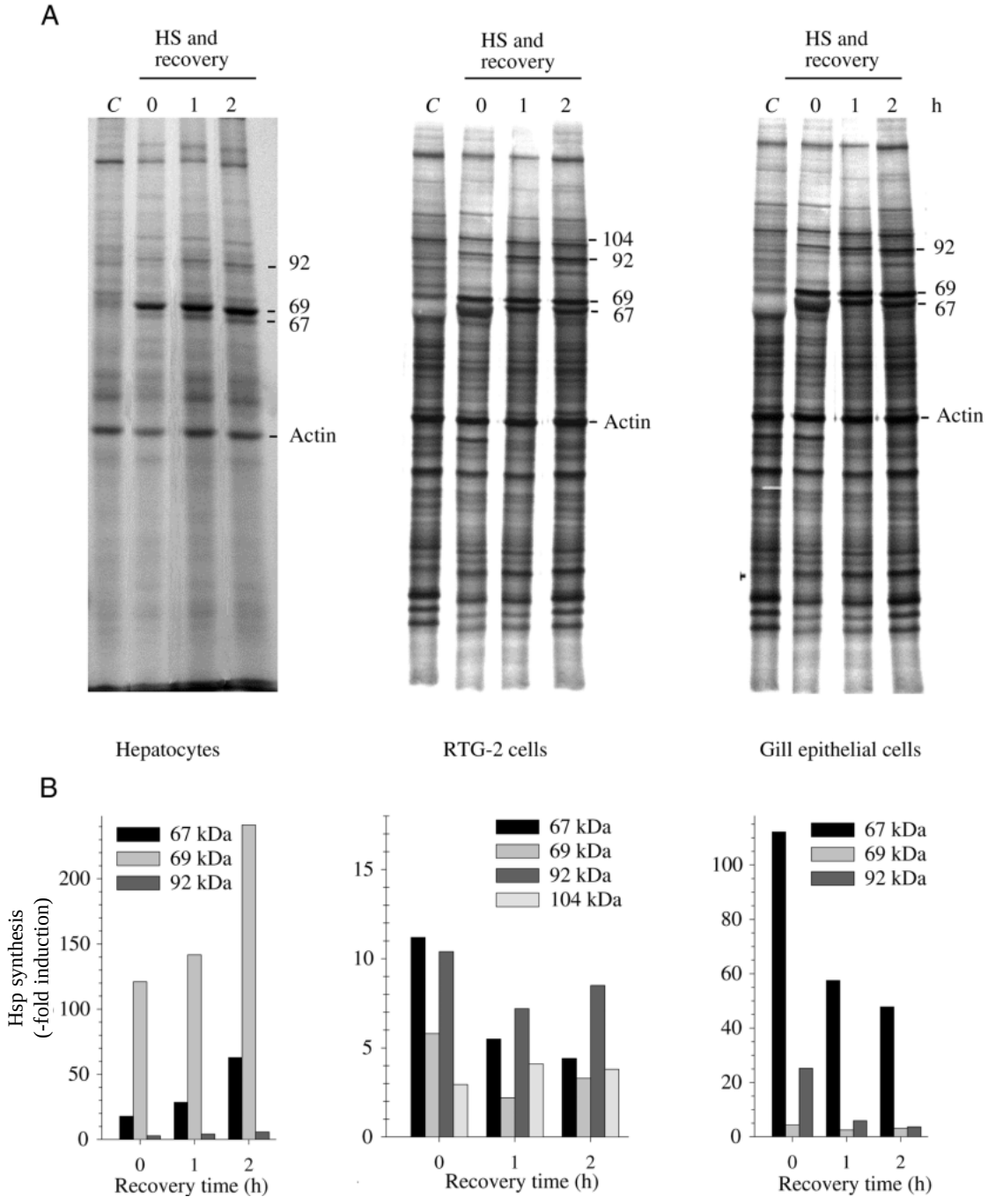

Fig. 1. Analysis of protein synthesis during heat shock (HS) by metabolic labelling. (A) Hepatocytes, RTG-2 cells and gill epithelial cells maintained at 18 °C were exposed to a temperature of 26 °C for 2 h. Recovery times (h) after the heat shock are indicated. The lanes marked with *C* indicate samples from untreated cells. The molecular masses of proteins (in kDa) were estimated from the molecular mass markers. (B) Quantitative analyses of the induction of synthesis of heat-shock proteins (Hsps) calculated on the basis of the Hsp/actin ratio. A value of 1 indicates that the level of synthesis was similar to that in the control samples. Values greater than 1 indicate increased synthesis of a protein. The synthesis of 67, 69 and 92 kDa proteins was significantly (P<0.05) (Wilcoxon matched-pairs signed-ranks test) increased in all treated cells and the synthesis of a 104 kDa protein was significantly (P<0.05) increased in RTG-2 cells.

factor similar to mammalian HSF1 or HSF2 by incubating the whole-cell extracts with murine anti-HSF1 and anti-HSF2 antibodies prior to the binding reaction. The binding of anti-HSF1 antibody to fish whole-cell extract caused a further reduction in electrophoretic mobility, i.e. a supershift, when the extract was incubated with the HSE oligonucleotide, indicating that the antibody recognizing HSF1 interacted with a fish protein forming a complex with HSE (Fig. 3C). In contrast, no effect was observed when antibodies against HSF2 were used. Our findings show that the induction of Hsp70 in response to heat shock observed in rainbow trout hepatocytes is associated with the DNA-binding activity of an HSF1-like protein.

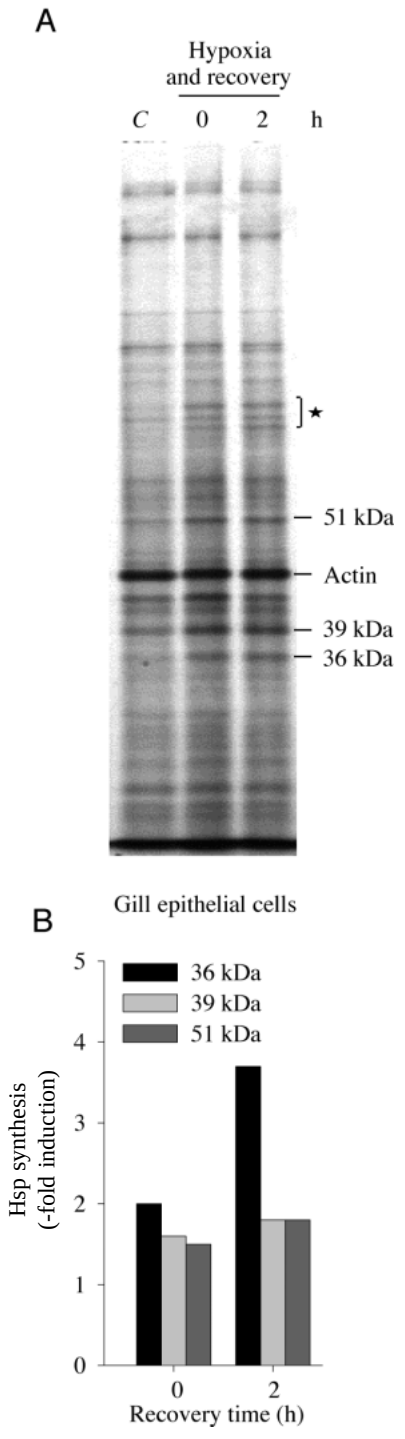

Fig. 2. Analysis of protein synthesis by metabolic labelling in gill epithelial cells after 24 h of hypoxia (1 % O_2). (A) Recovery times (h) after hypoxia are indicated. A star indicates proteins that probably represent the Hsp70 family. *C*, untreated cells. Quantitative analysis of the induced proteins (B) was carried out as in Fig. 1B. According to the Wilcoxon matched-pairs signed-ranks test, the synthesis of 36, 39 and 51 kDa proteins was increased (P<0.05) in hypoxia-treated cells.

Induction of hsp70 *mRNA expression*

To study the heat-shock response at the level of gene expression, we measured the amount of *hsp70* mRNA in heat-shocked fish hepatocytes. For the northern blot analysis, a probe specific for human *hsp70* was used. As shown in Fig. 4, we could detect mRNA corresponding to the fish *hsp70*, which

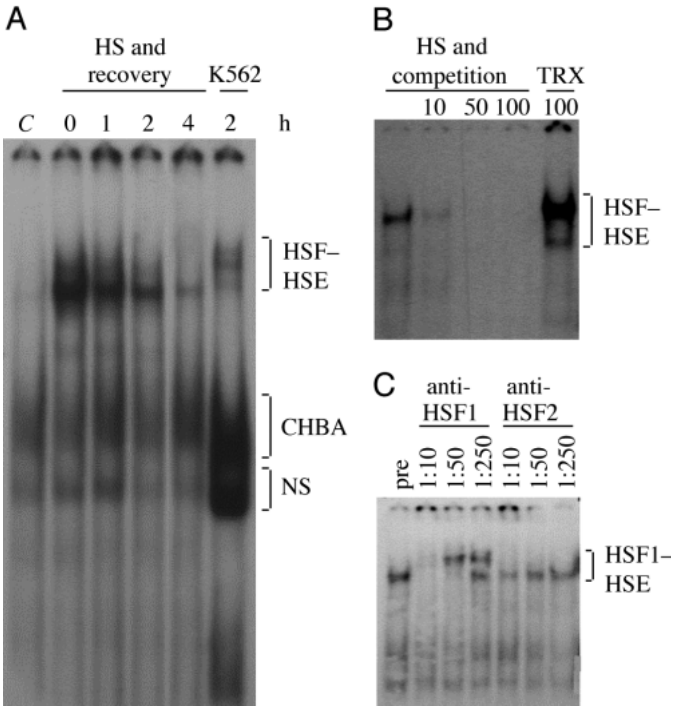

Fig. 3. (A) The effects of heat shock on heat-shock transcription factor (HSF) DNA-binding activity in hepatocytes. The recovery time (h) after the 2 h heat shock (HS) is indicated at the top of each lane. The locations of the specific HSF–HSE complex (HSF–HSE), the constitutive HSF DNA-binding activity (CHBA; Mosser *et al.* 1988) and the nonspecific complex (NS) are indicated on the right. Untreated cells are denoted by *C*, and the right-hand lane represents a positive heat-shock control from human erythroleukaemia K562 cells (K562). (B) Competition experiment with increasing concentrations of unlabelled HSE oligonucleotide (the molar excess is indicated at the top of each lane) using hepatocytes heat-shocked for 2 h. An oligonucleotide from the promoter region of the thioredoxin gene (TRX) was used as a non-specific oligonucleotide to indicate the specificity of the binding activity of a fish protein to *Drosophila* HSE. (C) Perturbation assay using heat-shocked (2 h) hepatocytes with anti-HSF1 and anti-HSF2 antibodies. Dilutions of HSF antibodies are indicated at the top of each lane. Preimmune serum (pre) was used as a control.

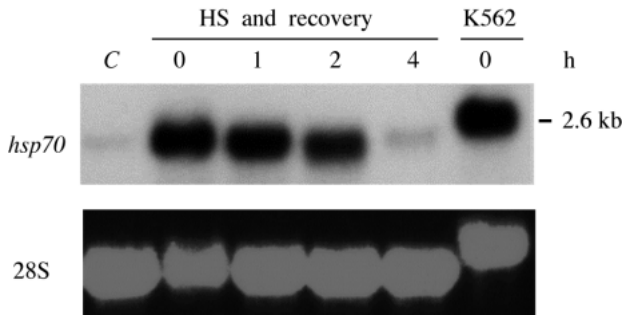

Fig. 4. Northern blot analysis of *hsp70* mRNA from control (*C*) and heat-shocked (HS, 26 °C for 2 h) hepatocytes. Recovery times (h) after heat shock are indicated at the top of each lane. The size of the mRNA in kilobases (kb) is indicated on the right. RNA from heat-shocked human K562 cells was used as a positive control (right-hand lane). The 28S ribosomal subunit (lower panel) was used as a control for equal loading.

was slightly smaller than the human *hsp70* mRNA. The amount of *hsp70* mRNA peaked immediately after the heat shock and returned to the control level within 4 h. Thus, there was a marked increase in *hsp70* gene expression in response to elevated temperature. Furthermore, the kinetics of activation of DNA binding and the increase in *hsp70* gene expression showed a remarkable correlation (Figs 3A, 4).

Discussion

Expression of heat-shock proteins

The temperature optimum for rainbow trout is between 10 and 15 °C, and the upper lethal temperature is close to the temperature, 26 °C (Schurmann *et al.* 1991), that we used to induce the heat-shock response in isolated cells. Under physiological conditions, all the cell types studied, hepatocytes, gill epithelial cells and gonadal fibroblasts, from which the RTG-2 cell line has been derived, are exposed to similar temperature changes. Correspondingly, the temperature response occurred in a similar manner regardless of cell type. Furthermore, our results are consistent with earlier studies on RTG-2 and CHSE (chinook salmon embryo) cells (Heikkila *et al.* 1982; Kothary and Candido, 1982) and on primary cultured catfish hepatocytes (Koban *et al.* 1987). Exposure to an elevated temperature induced the synthesis of proteins representing several Hsp families. Specifically, we observed increased synthesis of two Hsp70 proteins with molecular masses of 67 and 69 kDa and of proteins belonging to the Hsp90 and Hsp104 families.

The Hsp70 family consists of heat-inducible and constitutively expressed (heat-shock cognate) members. In the present study, the constitutive synthesis of Hsp70 appeared to be fairly low compared with the synthesis of other proteins under control conditions. A marked increase in synthesis occurred upon heat treatment. Only the overall synthesis of Hsp70 members was studied since there are no rainbow trout antibodies which specifically recognize the accumulation of the inducible Hsp70 forms. Commercially available Hsp70 antibodies which recognize both the constitutive and inducible members of the mammalian Hsp70 family gave a strong signal in western blots for both untreated and heat-shocked cells (S. Airaksinen, C. Råbergh, L. Sistonen and M. Nikinmaa, unpublished data), indicating that the constitutive levels of Hsp70 are fairly high despite the relatively low levels of Hsp synthesis under control conditions. In contrast to the constitutive members of the Hsp70 family, antibodies specifically designed against the inducible Hsp70 forms in mammals failed to recognize the pronounced synthesis of the 67 and 69 kDa proteins (S. Airaksinen, C. Råbergh, L. Sistonen and M. Nikinmaa, unpublished data). Similarly, some other studies have shown that Hsp70, which is recognized by various antibodies, is also present in large amounts under control conditions (Fader *et al.* 1994; Yu *et al.* 1994; Currie and Tufts, 1997). It has been shown recently that the polyclonal antibody specific for the trout Hsp70 family recognizes six constitutive and inducible forms, which may be separated by isoelectric focusing (Forsyth *et al.* 1997). It appears that the inducible Hsp70 forms have evolved more recently than their constitutive cognates (Sanders *et al.* 1994; Favatier *et al.* 1997).

In the present study, the induced synthesis of Hsps was clearly cell-type- and tissue-specific. Hsp104 was induced only in RTG-2 cells, and the intensity of induction of the 69 kDa protein compared with the 67 kDa protein was much greater in hepatocytes than in the other two cell types. Interestingly, Dietz and Somero (1993) have observed both tissue- and species-specificity of Hsp70 and Hsp90 synthesis in brain, gill and liver of four different fish species.

It should be noted that the protein expression pattern of cells taken from fish exposed to a stressor may differ from the response of stressed primary cell cultures or immortalized cell lines. For example, Kultz (1996) reported a different osmotic shock response in cultured gill epithelial cells and in gill cells of osmotically challenged fish. As stated by Ryan and Hightower (1994), the applicability of any *in vitro* results to the whole animal will be as good as the degree to which the culture system is representative of the tissue or organ from which it is derived.

Regulation of the heat-shock response in hepatocytes

Multiple transcription factors (HSFs) regulate Hsp synthesis in different organisms. Three distinct human HSFs have been isolated (Rabindran *et al.* 1991; Schuetz *et al.* 1991; Nakai *et al.* 1997). Among these, HSF1 is involved in the regulation of the classical heat-shock response (Baler *et al.* 1993; Sarge *et al.* 1993). Our results showed a pronounced heat stress responsive binding of an HSF1-related protein to the *Drosophila* heat-shock element. Thus, the regulation of Hsp synthesis in fish seems to conform to the mammalian pattern (Schlesinger, 1994), i.e. the heat-shock response is associated with the binding of an HSF1-like transcription factor to DNA. To determine whether an HSF1-like protein is present in fish, we have recently cloned from zebrafish (*Danio rerio*) the full-length HSF containing an open reading frame of 512 amino

acid residues. The fish HSF shows 60–70 % homology to mammalian HSF1 and 40 % homology to HSF2 (C. Råbergh, S. Airaksinen, A. Soitamo, H. Björklund, T. Johansson, L. Sistonen and M. Nikinmaa, unpublished data).

In the present study, we have not measured the transcription rate of *hsp70* directly. However, the expression of the *hsp70* gene is markedly facilitated by heat shock, since the amount of *hsp70* mRNA is significantly elevated in response to heat shock. Levels of *hsp70* mRNA are increased transiently, since maximum expression occurs immediately after heat shock and the mRNA levels return to control values within 4 h. The changes in mRNA levels correlated closely with the changes in the synthesis of the 69 kDa protein. This observation is in agreement with the suggestion of Currie and Tufts (1997) that Hsp70 induction in rainbow trout red blood cells is controlled primarily at the transcriptional level. Their suggestion is further supported by the close correlation between the activation of an HSF1-related factor and target gene expression observed in our study. The rapid decrease in *hsp70* mRNA levels during recovery could also be due to increased degradation. The complex regulation of heat-shock gene expression is reflected by increased stability of *hsp70* mRNA upon heat shock in human HeLa cells (Theodorakis and Morimoto, 1987). Similarly, *hsp70* mRNA has been shown to be stabilized in human chondrocytic cells exposed to high hydrostatic pressure (Kaarniranta *et al.* 1998).

Responses to hypoxia in gill epithelial cells

Although the heat-shock response is induced by a wide variety of stresses, among them hypoxia/anoxia, in mammalian cells (Zimmerman *et al.* 1991; Graven *et al.* 1993; Tucci *et al.* 1996; Graven and Farber, 1997), the hypoxic conditions employed in the present study caused a different response in terms of protein synthesis from that observed in response to heat shock. None of the three proteins, 36, 39 and 51 kDa, induced by hypoxia was induced by elevated temperature. The hypoxia response also showed a pronounced cell-type specificity, since it occurred in gill epithelial cells, which naturally experience large fluctuations in oxygen tensions, but not in hepatocytes or in RTG-2 cells. Land *et al.* (1993) observed that the synthesis of several proteins increased in hepatocytes of anoxic turtles. The difference between our results and those of Land *et al.* (1993) may be related to the difference in oxygen tensions utilized. In mammalian systems, it has been observed that protein synthesis under anoxic conditions differs from that under hypoxic conditions (Wenger and Gassmann, 1996). Alternatively, the differences between the anoxia-tolerant turtle and the hypoxia-intolerant rainbow trout may be related to differences in environmental requirements or to evolutionary distance between the animals. Clearly, much more information about hypoxia/anoxia-induced protein synthesis in non-mammalian vertebrates is required before generalizations can be made about the response.

Although the present results were not designed to identify the proteins induced by hypoxia, it is of interest to note that the size of these proteins correlates with certain mammalian proteins that are induced during hypoxia. Haem oxygenase (Bashan *et al.* 1992) has a molecular mass of 32 kDa, erythropoietin a molecular mass of 34 kDa (Goldberg *et al.* 1987), glucose transporter-1 a molecular mass of 43 kDa (Sweiki *et al.* 1992) and lactate dehydrogenase a molecular mass of 36 kDa (Firth *et al.* 1994). Interestingly, Almeida-Val *et al.* (1995) have shown that hypoxia alters the isoenzyme pattern of lactate dehydrogenase in *Cichlasoma amazonarum* in a tissue-dependent manner. Thus, the tissue specificity of hypoxia-induced protein synthesis may be a common phenomenon in fish.

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